RESEARCH ARTICLE

Biological characteristics of dynamic expression of nerve regeneration related growth factors in dorsal root ganglia after peripheral nerve injury

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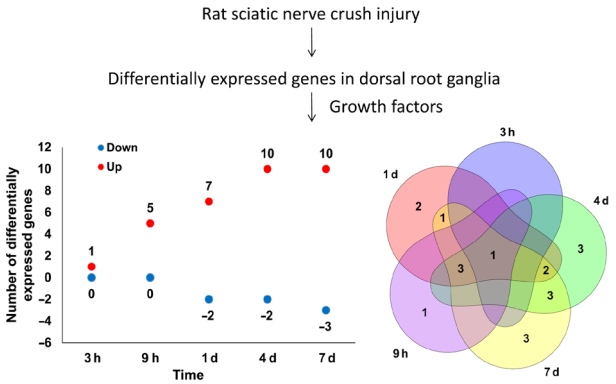
Graphical Abstract

Dynamic changes of growth factors in rat dorsal root ganglia after sciatic nerve crush injury



Time	Elements	Total	Time	Elements	Total
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1 d	Inhbb Lefty1	2	4 d, 7 d	Gdnf Nog Agt	3
4 d	Fgf19 Amelx Nrg4	3	1 d, 4 d, 7 d	Ngf Bdnf	2
7 d	Fgf23 Vgf Dkk1	3	9 h, 1 d, 4 d, 7 d	Nodal Fgf2 Areg	3
			3 h, 9 h, 1 d, 4 d, 7 d	Lep	1

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Abstract

The regenerative capacity of peripheral nerves is limited after nerve injury. A number of growth factors modulate many cellular behaviors, such as proliferation and migration, and may contribute to nerve repair and regeneration. Our previous study observed the dynamic changes of genes in L4–6 dorsal root ganglion after rat sciatic nerve crush using transcriptome sequencing. Our current study focused on upstream growth factors and found that a total of 19 upstream growth factors were dysregulated in dorsal root ganglions at 3, 9 hours, 1, 4, or 7 days after nerve crush, compared with the 0 hour control. Thirty-six rat models of sciatic nerve crush injury were prepared as described previously. Then, they were divided into six groups to measure the expression changes of representative genes at 0, 3, 9 hours, 1, 4 or 7 days post crush. Our current study measured the expression levels of representative upstream growth factors, including nerve growth factor, brain-derived neurotrophic factor, fibroblast growth factor 2 and amphiregulin genes, and explored critical signaling pathways and biological process through bioinformatic analysis. Our data revealed that many of these dysregulated upstream growth factors, including nerve growth factor, brain-derived neurotrophic factor, fibroblast growth factor 2 and amphiregulin, participated in tissue remodeling and axon growth-related biological processes Therefore, the experiment described the expression pattern of upstream growth factors in the dorsal root ganglia after peripheral nerve injury. Bioinformatic analysis revealed growth factors that may promote repair and regeneration of damaged peripheral nerves. All animal surgery procedures were performed in accordance with Institutional Animal Care Guidelines of Nantong University and ethically approved by the Administration Committee of Experimental Animals, China (approval No. 20170302-017) on March 2, 2017.

Key Words: *axon growth; bioinformatic analysis; dorsal root ganglia; growth factors; Ingenuity Pathway Analysis; nerve regeneration; peripheral nerve injury; rat sciatic nerve crush injury; transcriptome sequencing; upstream regulators*

Chinese Library Classification No. *R459.9; R363; R364*

Introduction

Peripheral nerve injury is a widespread medical problem worldwide (Sung et al., 2019). It is reported that around 2.8% of trauma patients suffer from peripheral nerve injury (Noble et al., 1998). In developed countries, peripheral nerve injury affects approximately 13 to 23 per 100,000 persons per year (Li et al., 2014). The peripheral nervous system has some regeneration ability after nerve injury. However, the regeneration rate of injured axons is slow and the functional recovery effects are generally not very satisfactory (Gordon et al., 2009; Chan et al., 2014; Gu, 2015). Achieving a better understanding of the genetic changes after peripheral nerve injury will help the discovery of essential factors for peripheral nerve regeneration and provide certain cues for the treatment of nerve injury.

Schwann cells in the peripheral nervous system contribute to the construction of a permissive and favorable microenvironment for peripheral nerve regeneration (Namgung, 2014; Jessen and Mirsky, 2016; Carr and Johnston, 2017). Schwann cells switch to a dedifferentiated state, proliferate and migrate to build the bands of Bungner, and then redifferentiate to form myelin sheath around regenerated axons (Ohara and Ikuta, 1988; Frostick et al., 1998). During this process, many growth factors, including nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF), and brain-derived neurotrophic factor (BDNF), stimulate the proliferation, migration, or re-myelination of Schwann cells that benefits the regeneration of injured peripheral nerves (Li et al., 2015; Qin et al., 2016; Yi et al., 2016). The significance of growth factors in modulating Schwann cell phenotypes led to the identification of those key growth factors that were differentially expressed in rat sciatic nerve stumps after sciatic nerve injury (Zhang et al., 2019).

Besides the essential roles in Schwann cells, growth factors are also involved in the physiological and pathological conditions of neurons, such as neuronal survival, death, differentiation, morphology, and outgrowth (Lowenstein and Arsenault, 1996; Zawada et al., 1996; Whitmire et al., 2011; Schwieger et al., 2015; Onger et al., 2017). Therefore, growth factors, especially differentially expressed growth factors in neurons after nerve injury, may regulate neuronal behaviors, activate the intrinsic growth capacity of neurons, and promote axon regrowth.

To identify essential growth factors during peripheral nerve degeneration and regeneration, we screened differentially expressed genes in dorsal root ganglia (DRG) after rat sciatic nerve injury using previously obtained transcriptome sequencing data (Gong et al., 2016). We explored upstream regulators of these differentially expressed genes with Ingenuity Pathway Analysis software, and screened dysregulated upstream growth factors. Gene Ontology (GO) category analysis and Kyoto Enrichment of Genes and Genome (KEGG) pathway analysis were then applied to identify biologically relevant pathways and processes.

Materials and Methods

Animals

Thirty-six adult male Sprague-Dawley rats, weighing 180–220 g, were obtained from Experimental Animal Center of Nantong University, China (animal licenses No. SCXK [Su] 2014-0001 and SYXK [Su] 2012-0031). All animal surgery procedures were performed in accordance with Institutional Animal Care Guidelines of Nantong University and ethically approved by the Administration Committee of Experimental Animals, China (approval No. 20170302-017) on March 2, 2017.

Animal surgery and tissue collection

Sprague-Dawley rats were subjected to sciatic nerve crush injury according to a standardized surgical method (Luis et al., 2008) with modifications as previously described (Yi et al., 2015). Following anesthetization, rat sciatic nerve stumps were exposed at 10 mm above the bifurcation into the tibial and common fibular nerves and crushed three times, each for 10 seconds, with forceps. Rats were randomly divided into six groups and sacrificed by cervical dislocation at 0, 3, 9 hours, 1, 4, or 7 days after surgery. The lumbar 4, 5 and 6 (L4–6) DRGs were removed from each Sprague-Dawley rat in each groups, collected, and stored at –80°C.

Bioinformatic analysis

Transcriptome sequencing of DRGs at 0, 3, 9 hours, 1, 4, or 7 days after surgery was conducted as described previously (Gong et al., 2016). Raw data were stored in the online database (SRP200823, PRJNA547681) (https://www.ncbi.nlm.nih.gov/sra/SRP200823). Gene expression levels in DRGs at 3, 9 hours, 1, 4, or 7 days after rat sciatic nerve crush injury were determined by reads per kilobase transcriptome per million mapped reads method and compared with their expressions at zero hour. Relative gene expression patterns were core analyzed with Ingenuity Pathway Analysis software (Ingenuity Systems Inc., Redwood City, CA, USA) with a set cutoff of $\log_2$ ratio < –1 or > 1 and experimental false discovery rate (q-value) < 0.05.

Ingenuity Pathway Analysis upstream analysis was applied to identify the involved growth factors in DRGs at each time point after rat sciatic nerve crush injury by selecting growth factors among upstream regulators. Genes coding for growth factors with a $\log_2$ ratio < –1 or > 1 at 3, 9 hours, 1, 4, or 7 days as compared with zero hour were screened and considered as dysregulated upstream growth factors. These identified growth factors in DRGs at each time point were determined and enriched GO categories and KEGG pathways were identified using Database for Annotation, Visualization, and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/).

RNA isolation and polymerase chain reaction validation

Total RNA samples were isolated from rat L4–6 DRGs and collected at each time point after surgery, with TRIzol reagent (Life Technologies, Carlsbad, CA, USA). RNA samples were reverse transcribed to cDNA using the Prime-Script reagent kit (TaKaRa, Dalian, Liaoning, China). The expression levels of the target genes were determined by polymerase chain reaction using SYBR Premix Ex Taq (TaKaRa) on an Applied Biosystems Step One System (Applied Biosystems, Foster City, CA, USA). The relative expression patterns of

target genes in DRGs at different points after sciatic nerve crush injury were measured using the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with *Gapdh* as the reference gene. The sequences of primer pairs of target genes and reference gene are listed in **Table 1**.

Table 1 Primer sequences of validated genes

Gene		Sequence (5′–3′)	Product size (bp)
Ngf	Forward	CCA AGG ACG CAG CTT TCT ATC	186
	Reverse	CTG TGT CAA GGG AAT GCT GAA G	
Bdnf	Forward	AAC CAT AAG GAC GCG GAC TT	222
	Reverse	TGC AGT CTT TTT ATC TGC CG	
Fgf2	Forward	ACT TCA AGG ATC CCA AGC GG	182
	Reverse	CAT AGC CAG GTA CCG GTT CG	
Areg	Forward	CCA ATG AGA ACT CCG TCG CT	157
	Reverse	AAA CCA CAA GTC CAC CAG CA	
Gapdh	Forward	ACA GCA ACA GGG TGG TGG AC	252
	Reverse	TTT GAG GGT GCA GCG AAC TT	

Areg: Amphiregulin; *Bdnf*: glial cell line-derived neurotrophic factor; *Fgf2*: fibroblast growth factor 2; *Gapdh*: glyceraldehyde-3-phosphate dehydrogenase; *Ngf*: nerve growth factor.

Statistical analysis

Polymerase chain reaction results were summarized from three paired experiments and presented as the mean ± SEM. One-way analysis of variance followed by Dunnett's *post hoc* test was applied for statistical comparison using GraphPad Prism 6.0 software (GraphPad Software, Inc., San Diego, CA, USA). A P value < 0.05 was considered statistically significant.

Results

Discovery of dysregulated upstream growth factors in DRGs after sciatic nerve injury

Previously, the global gene expression in DRGs after peripheral nerve injury was determined by preparing rat models of sciatic nerve crush injury, collecting DRGs and performing transcriptome sequencing (Gong et al., 2016). In view of the importance of growth factors in regulating neuron activities and nerve regeneration, dysregulated genes with log2 ratio < –1 or > 1 and q-value < 0.05 were further core analyzed by Ingenuity Pathway Analysis software to screen upstream growth factors of dysregulated genes. Dysregulated upstream growth factors were then selected and identified.

As compared with the zero hour control, 1, 5, 9, 12, and 13 dysregulated upstream growth factors were identified in DRGs at 3, 9 hours, 1, 4, and 7 days after sciatic nerve crush injury, respectively (**Figure 1A**). The majority of these differentially expressed upstream growth factors were up-regulated after sciatic nerve crush injury (**Figure 1A**). The Venn diagram shows that one specific growth factor, leptin (*Lep*), was persistently differentially expressed in DRGs at all tested time points after sciatic nerve crush injury (**Figure 1B** & **C**). Nodal growth differentiation factor (*Nodal*), *Fgf2*, and amphiregulin Areg were all dysregulated in DRGs at 9 hours, 1, 4, and 7 days after injury. *Ngf* and *Bdnf* were dysregulated in DRGs at 1, 4, and 7 days after injury. *Gdnf*, noggin (*Nog*), and angiotensinogen (*Agt*) were dysregulated in DRGs at both 4 and 7 days after injury. Nudix hydrolase 6 (*Nudt6*) was dysregulated in DRGs at both 1 and 7 days after injury. Other growth factors, including epiregulin (*Ereg*), inhibin subunit beta B (*Inhbb*), left-right determination factor (*Lefty-1*), fibroblast growth factor 19 (*Fgf19*), X chromosome linked amelogenin (*Amelx*), neuregulin 4 (*Nrg4*), fibroblast growth factor 23 (*Fgf23*), Vgf nerve growth factor inducible (*Vgf*), and dickkopf-1 (*Dkk1*), a WNT signaling pathway inhibitor, were only dysregulated in DRGs at one single time point after injury.

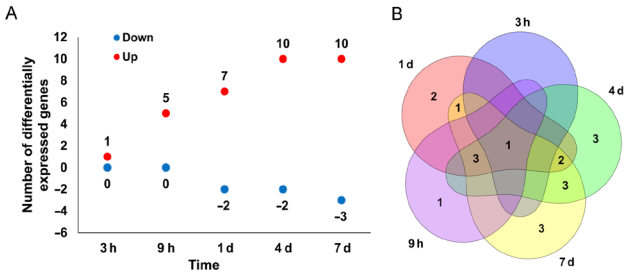
Dynamic changes of representative dysregulated upstream growth factors

A precise determination of gene expression levels laid the foundation for subsequent bioinformatic analysis. To validate the veracity of sequencing outcomes, the DRGs of sciatic nerve crushed rats were collected and the expression levels of representative differentially expressed upstream growth factors were measured by polymerase chain reaction. Two representative upstream growth factors that were commonly dysregulated in DRGs at 1, 4 and 7 days after injury, *Ngf* and *Bdnf*, and two representative upstream growth factors that were commonly dysregulated in DRGs at 9 hours, 1, 4 and 7 days after injury, *Fgf2* and *Areg*, were selected for validation (**Figure 2**).

As compared with the zero hour control, the gene expression levels of *Ngf* in DRGs reached about 8-fold at 1 day after nerve injury and the expression levels remained high at later time points (**Figure 2A**). However, the gene expression levels of *Bdnf* only increased at 1 day after nerve injury (**Figure 2B**). The gene expression levels of *Fgf2* slightly increased at 9 hours and then significantly increased at 1, 4 and 7 days (**Figure 2C**). The gene expression levels of *Areg* were also upregulated in DRGs after sciatic nerve crush injury (**Figure 2D**). The polymerase chain reaction results were generally consistent with the transcriptome sequencing data, indicating the accuracy of transcriptome sequencing.

Bioinformatic analysis of dysregulated upstream growth factors in DRGs

The global temporal expression patterns of differentially expressed upstream growth factors in DRGs after sciatic nerve injury were displayed in a heatmap and clustered (**Figure 3A**). The heatmap showed that these growth factors could be mainly grouped into two clusters: at 0–9 hours and 1–7 days (**Figure 3A**). Bioinformatic analysis using the DAVID database identified the top 10 significantly involved GO categories (**Figure 3B**) and KEGG pathways (**Figure 3C**).



Time	Elements	Total	Time	Elements	Total
9 h	Ereg	1	1 d, 7 d	Nudt6	1
1 d	Inhbb Lefty1	2	4 d, 7 d	Gdnf Nog Agt	3
4 d	Fgf19 Amelx Nrg4	3	1 d, 4 d, 7 d	Ngf Bdnf	2
7 d	Fgf23 Vgf Dkk1	3	9 h, 1 d, 4 d, 7 d	Nodal Fgf2 Areg	3
			3 h, 9 h, 1 d, 4 d, 7 d	Lep	1

Figure 1 Overview of dysregulated upstream growth factors in dorsal root ganglions at 3, 9 hours, 1, 4, and 7 days after sciatic nerve crush injury.

(A) Number of dysregulated upstream growth factors in dorsal root ganglions. Red shows up-regulated upstream growth factors and blue shows down-regulated upstream growth factors. (B) Overlap of dysregulated upstream growth factors. The different colors represent dysregulated growth factors at different days (3 h, 9 h, 1 d, 4 d, 7 d) in Venn diagram. (C) Gene list of dysregulated upstream growth factors. 1, 2, 3 indicate the number of dysregulated growth factors. *Agt*: Angiotensinogen; *Amelx*: amelogenin X-linked; *Areg*: amphiregulin; *Bdnf*: brain derived neurotrophic factor; *Dkk1*: dickkopf WNT signaling pathway inhibitor 1; *Ereg*: epiregulin; *Fgf19*: fibroblast growth factor 19; *Fgf2*: fibroblast growth factor 2; *Fgf23*: fibroblast growth factor 23; *Gdnf*: glial cell line-derived neurotrophic factor; *Inhbb*: inhibin subunit beta B; *Lefty*: left-right determination factor; *Lep*: leptin; *Ngf*: nerve growth factor; *Nodal*: nodal growth differentiation factor; *Nog*: noggin; *Nrg4*: neuregulin 4; *Nudt6*: nudix hydrolase 6; *Vgf*: Vgf nerve growth factor inducible.

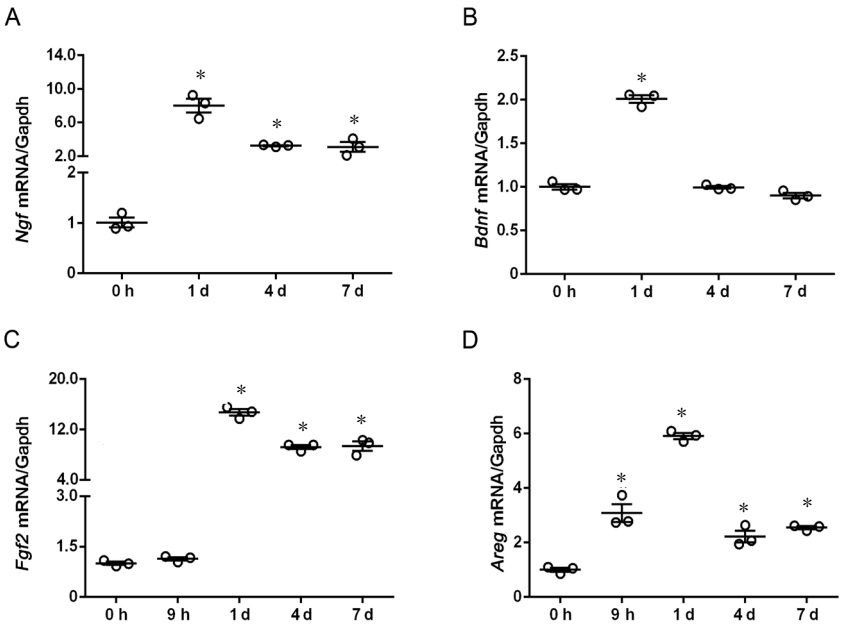


Figure 2 Dynamic changes of *Ngf*, *Bdnf*, *Fgf2*, and *Areg* in dorsal root ganglions after sciatic nerve crush injury.

(A–D) Gene abundances of *Ngf*, *Bdnf*, *Fgf2*, and *Areg* in dorsal root ganglions at 0, 4, and 7 days after sciatic nerve crush injury: the Y-axis indicates the relative expression levels of target genes *Ngf*, *Bdnf*, *Fgf2*, and *Areg* to that of *Gapdh*. The abundances of target genes were normalized with zero hour control. $*P < 0.05$, *vs.* zero hour. *Areg*: Amphiregulin; *Bdnf*: brain derived neurotrophic factor; *Fgf2*: fibroblast growth factor 2; *Gapdh*: glyceraldehyde-3-phosphate dehydrogenase; *Ngf*: nerve growth factor.

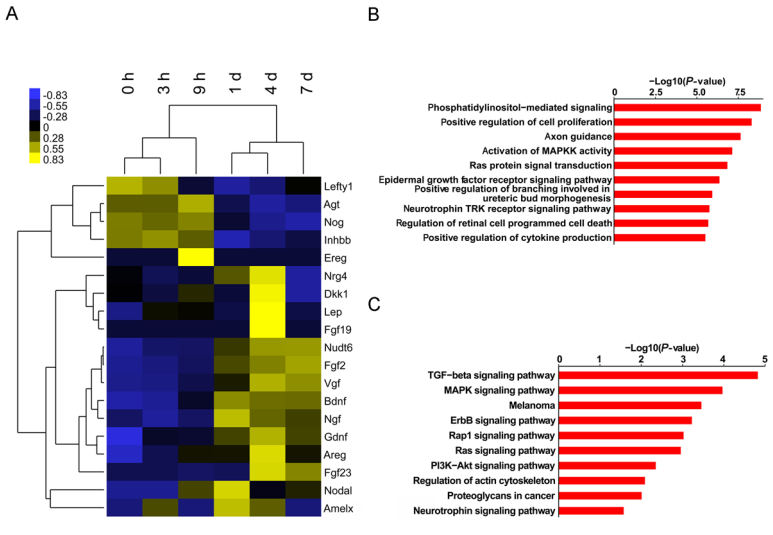


Figure 3 Bioinformatic analysis of dysregulated upstream growth factors in dorsal root ganglions after sciatic nerve crush injury.

(A) Heatmap of dysregulated upstream growth factors in dorsal root ganglions: Yellow shows up-regulated upstream growth factors and blue shows down-regulated upstream growth factors. (B) Enriched top 10 Gene Ontology (GO) categories of dysregulated upstream growth factors in dorsal root ganglions. (C) Enriched top 10 Kyoto Enrichment of Genes and Genome (KEGG) pathways of dysregulated upstream growth factors in dorsal root ganglions: Yellow shows up-regulated upstream growth factors and blue shows down-regulated upstream growth factors. *Agt*: Angiotensinogen; *Amelx*: amelogenin X-linked; *Areg*: amphiregulin; *Bdnf*: brain derived neurotrophic factor; *Dkk1*: dickkopf WNT signaling pathway inhibitor 1; *Ereg*: epiregulin; *Fgf19*: fibroblast growth factor 19; *Fgf2*: fibroblast growth factor 2; *Fgf23*: fibroblast growth factor 23; *Inhbb*: inhibin subunit beta B; *Lefty*: left-right determination factor; *Lep*: leptin; *Ngf*: nerve growth factor; *Nodal*: nodal growth differentiation factor; *Nog*: noggin; *Nrg4*: neuregulin 4; *Nudt6*: nudix hydrolase 6; *Vgf*: Vgf nerve growth factor inducible.

Given that our focus was axon regrowth during peripheral nerve repair and regeneration, GO biological processes that were highly related to organ remodeling and tissue regeneration were specifically investigated (**Figure 4A**). These tissue regeneration-related GO biological processes could be divided into two groups: before day one and after day one. This suggested that the biological processes occurring in the DRGs might be different in the acute phase and the sub-acute phase after nerve injury. As well as the overview of tissue regeneration-related GO biological processes, there were dynamic changes of growth factors in many essential biological processes. The following were displayed in **Figure 4B**; axon extension, axon guidance, axon target recognition, dendrite extension, cell migration involved in sprouting angiogenesis, brain-derived neurotrophic factor signaling pathway, glial cell proliferation, growth, negative regulation of neuron apoptotic process, nerve growth factor processing, nerve growth factor signaling pathway, neuron projection extension, regulation of axon extension, neuron recognition, and regulation of neurotransmitter secretion. It appeared that many essential biological processes also showed different trends during the first 24 hours after nerve injury.

Discovery of dysregulated upstream growth factors related to axon growth

The axons of neurons are damaged following severe peripheral nerve injury. Therefore axon growth is crucial for peripheral nerve regeneration and subsequent target reinnervation. In this study we further investigated dysregulated growth factors in DRGs after nerve injury and focused on growth factors that were involved the biological processes related with axon growth. These included axonogenesis, axon extension, axon guidance, cell growth, proliferation, migration, differentiation, and extracellular matrix remodeling and assembly.

Fourteen dysregulated upstream growth factors participated in these biological processes (**Figure 5A**). Ten of these 14 upstream growth factors, including *Fgf2*, *Bdnf*, *Fgf19*, *Nrg4*, *Fgf23*, *Amelx*, *Ngf*, *Nodal*, *Areg*, and *Gdnf*, were up-regulated, while the other 4 upstream growth factors, including *Lefty1*, *Nog*, *Inhbb*, and *Agt*, were down-regulated (**Figure 5A**). The expressions of these upstream growth factors in DRGs at each time point after nerve injury were assessed and the details shown in **Figure 5B–F**. Many upstream growth factors showed altered expression at 1 day after nerve injury, indicating that this might be the important time point for initiating axon growth.

Discussion

It is now possible to detect the expression levels of tens of thousands of transcripts in diverse physiological and pathological activities with the development of large-scale high-throughput data analysis methods, such as transcriptome sequencing and DNA array-based expression profiling (Nagalakshmi et al., 2010; Trapnell et al., 2010). To determine gene changes after peripheral nerve injury, the rat sciatic nerve injury model was chosen because rats have similar nerve trunk distributions as humans and their sciatic nerves are the largest peripheral nerve trunks in animals (Bryan et al., 1999; Magill et al., 2007; Savastano et al., 2014; Geuna, 2015). Gene expressions in DRGs after rat sciatic nerve injury, for example sciatic nerve chronic constriction injury and crush injury, have been comprehensively examined (Ha et al., 2001; Kim et al., 2001; Bosse et al., 2006; Martin et al., 2019). In a previous study, rat DRGs were collected at 0, 3, 9 hours, 1, 4, and 7 days after sciatic nerve crush injury for transcriptome sequencing to explore gene changes in DRGs and to screen altered genes during peripheral nerve degeneration and regeneration (Gong et al., 2016). In addition, differentially expressed transcription factors, especially transcription factors involved in cell death, have been discovered (Qin et al., 2018).

Growth factors are indispensable regulatory molecules for wound healing and tissue regeneration (Hajimiri et al., 2015; Park et al., 2017; Yamakawa and Hayashida, 2019). In the nervous system, growth factors play significant roles in modulating neuron behaviors and have been used for the treatment of many nervous system diseases, such as traumatic brain injury, amyotrophic lateral sclerosis and familial frontotemporal dementia (Suzuki and Svendsen, 2008; Seib et al., 2013; Petkau and Leavitt, 2014; Zhou et al., 2018). In the present study, 19 dysregulated upstream critical growth factors in DRGs were identified by re-analyzing previously obtained transcriptome sequencing data.

Many growth factors, including *Nudt6*, *Gdnf*, *Nog*, *Agt*, *Ngf*, *Bdnf*, *Nodal*, *Fgf2*, *Areg*, and *Lep* were dysregulated in DRGs at multiple time points after nerve injury. Many of these differentially expressed growth factors had been closely linked with neuron functions. For instance, NGF and BDNF promoted the neurite outgrowth as well as the survival of DRG neurons (Tosaki et al., 2008; Li et al., 2016; Castillo et al., 2018; Kaval Oguz, 2018). Besides these growth factors, the biological effects of many other commonly differentially expressed upstream growth factors on neurons were not fully investigated. It has been found that *Nog* expression inhibited BMP4-WNT1 signaling and neural crest cells delamination (Osorio et al., 2009). AGT was expressed in both astrocytes and neurons in the central nervous system of mice (Yang et al., 1999) and could affect the firing rates of neurons (Modgil et al., 2012). AREG regulated the autocrine survival of adult sensory neurons (Nilsson and Kanje, 2005). Our study showed that these growth factors were robustly changed after nerve injury and indicated that these growth factors might regulate neuron activities and contribute to peripheral nerve repair and regeneration. However, it was worth noting that DRGs contain multiple cell types besides neurons, such as perineuronal satellite glial cells (Castillo et al., 2013). Therefore, these dysregulated growth factors might not only be associated with DRG neurons but also with glial cells in DRGs. To evaluate the specific changes of genes in DRG neurons, single cell sequencing could be conducted in future studies. Moreover, our current investigation focused on the early period after peripheral nerve injury. To obtain a better view of dynamic changes of genes during the whole degeneration

and regeneration, gene profiling at longer time points, for instance 14, 21, or 28 days after sciatic nerve injury, should be further determined.

In conclusion, we have provided a general view of the dynamic profiling of dysregulated expressed upstream growth factors and discovered critical growth factors that might be potential targets for peripheral nerve injury treatment.

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Peer review: *Externally peer reviewed.*

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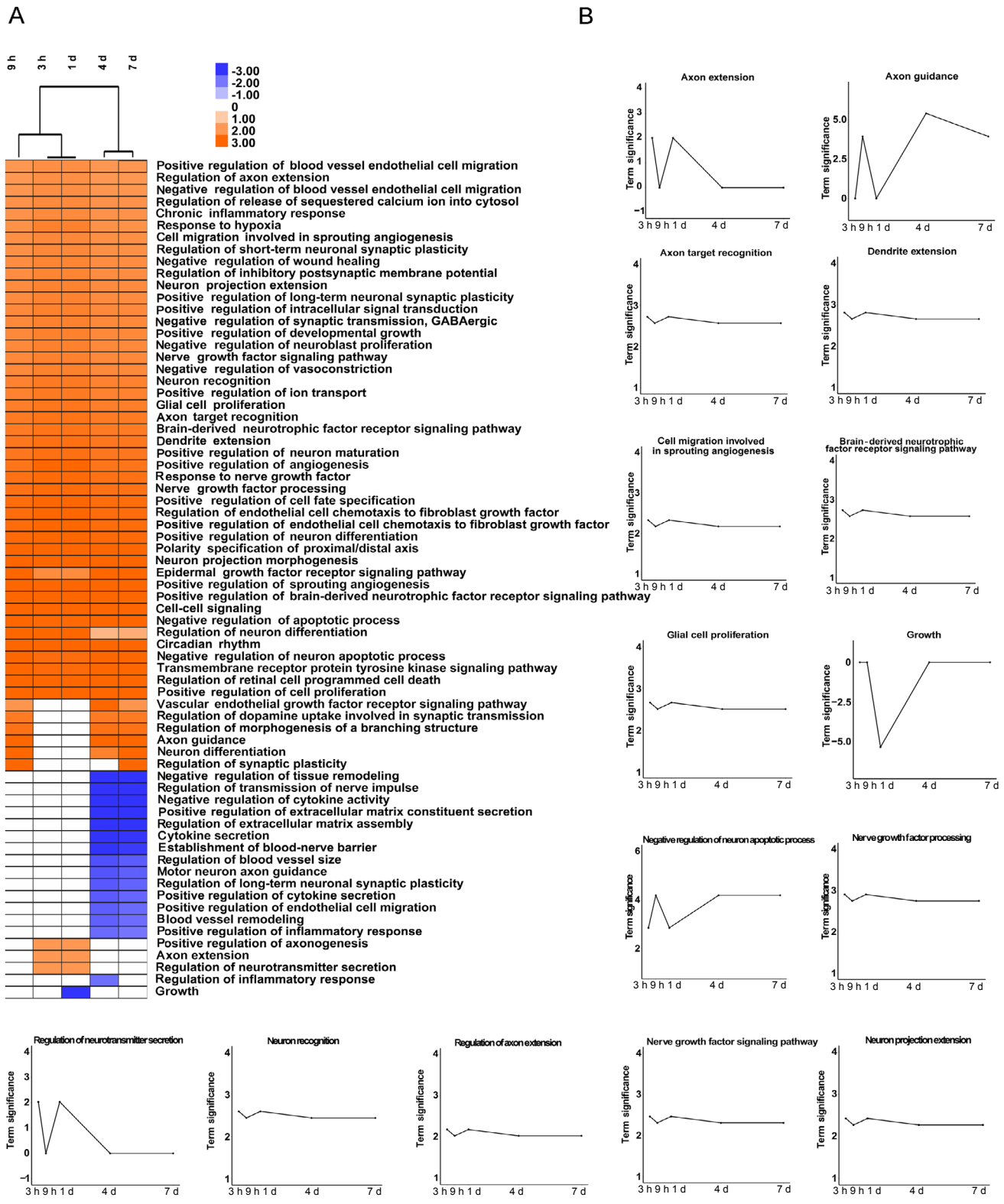

Figure 4 GO biological process categories in regeneration after sciatic nerve crush injury.
(A) Heatmap of GO biological process categories in regeneration: Orange shows up-regulated upstream growth factors and blue shows down-regulated upstream growth factors. (B) Temporal expression patterns of dysregulated upstream growth factors in specific GO biological process categories.

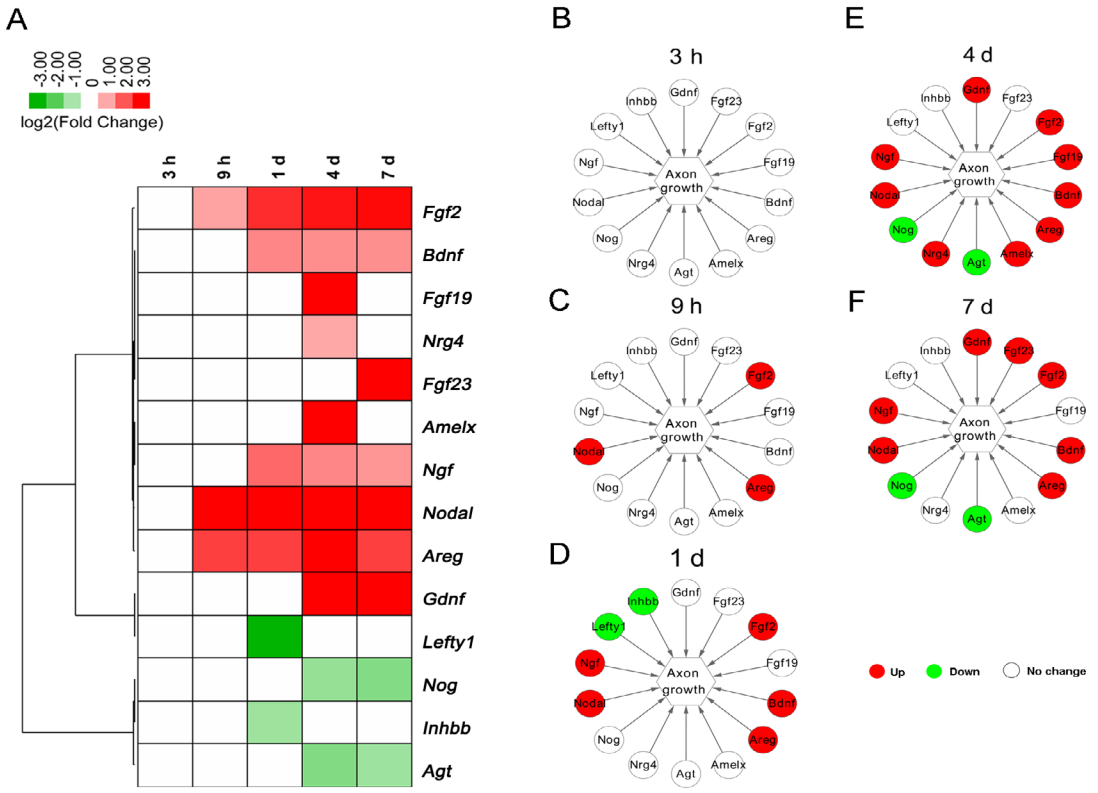

Figure 5 Dysregulated upstream growth factors involved in axon growth after sciatic nerve crush injury.
(A) Heatmap of dysregulated upstream growth factors involved in axon growth. (B–F) Changes in expression of growth factors at (B) 3 hours, (C) 9 hours, (D) 1 day, (E) 4 days, and (F) 7 days after surgery. Red shows up-regulated upstream growth factors and green shows down-regulated upstream growth factors. *Agt*: Angiotensinogen; *Amelx*: amelogenin X-linked; *Areg*: amphiregulin; *Bdnf*: brain derived neurotrophic factor; *Fgf19*: fibroblast growth factor 19; *Fgf2*: fibroblast growth factor 2; *Fgf23*: fibroblast growth factor 23; *Gdnf*: glial cell line-derived neurotrophic factor; *Inhbb*: inhibin subunit beta B; *Lefty*: left-right determination factor; *Ngf*: nerve growth factor; *Nodal*: nodal growth differentiation factor; *Nog*: noggin; *Nrg4*: neuregulin 4.

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